DECOMPOSITION OF CHITIN BY MARINE BACTERIA

CHARLES W. HOCK

(*From the Woods Hole Oceanographic Institution*,[1] *Woods Hole, Mass.*)

Chitin is an important constituent of the exoskeletons of many marine animals. For example, the shell of the horseshoe crab, *Limulus polyphemus*, consists on an average of 25 per cent chitin. Due to the death of marine inhabitants, and to the production of chitinous casts during the developmental stages of certain species, large quantities of chitin are released annually. If this material were not decomposed, much carbon and nitrogen would be withheld from the cycles of these elements in the sea. Analyses of marine sediments indicate, however, that relatively little chitin accumulates there; it must, therefore, be broken down into simpler chemical compounds, or mineralized.

Decomposition of chitin by mixed or pure cultures of marine bacteria has been demonstrated by several investigators (2, 4, 12, 14, 16, 17), whose observations indicate that chitin-decomposing bacteria have a worldwide distribution in the sea. The present investigation was undertaken to study the distribution of these bacteria in a relatively restricted region of the sea, and to follow the breakdown of chitinous materials in natural media from this same locality.

Chitin has an elementary composition of $C_{32}H_{54}O_{21}N_4$ (6). It is a tough leathery substance, insoluble in water, in concentrated alkalis and in the usual organic solvents. By hydrolysis with concentrated acid it yields glucosamine and acetic acid in proportions which indicate that there is an acetyl group for each glucosamine residue. So far no essential differences have been found in the chitin from animal or vegetable sources (5, 7).

Isolation of Chitin-Decomposing Bacteria

Purified chitin, used in making culture media for the detection and isolation of chitin-decomposing bacteria, was prepared from horseshoe crab shells by the methods of Benton (1) and ZoBell and Rittenberg (17). The shells were treated successively, for prolonged periods, with 1 per cent hydrochloric acid, 2 per cent potassium hydroxide and 95 per cent ethyl alcohol. The snowy-white material which remained reacted positively to simple qualitative tests for chitin (3).

[1] Contribution No. 231.

Liquid culture media were prepared by partly covering 1 × 5 cm. strips of purified chitin, in test tubes, with sea water, or with sea water containing 0.1 per cent concentrations of additional nutrients such as dextrose, peptone, or ammonium chloride. In some cases a small amount (0.005 per cent) of phosphate was added to the sea water to eliminate any effect due to lack of available phosphorus. The medium best suited for the detection of chitin-decomposing bacteria consisted of a strip of chitin partly covered with a solution used by Waksman (14), and having the following composition:

Glucose	1 gram
Peptone	1 gram
K_2HPO_4	0.05 gram
Sea water (filtered)	1,000 ml.

Solid media were prepared from the liquid culture media either by the addition of 15 grams of agar to each liter of solution or by diffusion of the nutrient solutions through silica gels. Where solid media were employed strips of sterile purified chitin were placed on the surface of the hardened gel. The silica gel plates were prepared under as nearly aseptic conditions as possible; all other media were sterilized by autoclaving for 20 minutes at 120° C.

One additional solid medium was found useful for the rapid detection of chitin-decomposing bacteria. It was prepared by uniformly dispersing chitin in agar according to the following procedure. Fifteen grams of purified chitin were added to 150 ml. of 1 : 1 sulfuric acid and kept overnight in the refrigerator. The chitin was dissolved by this treatment but analyses showed that it was not altered chemically, i.e. hydrolyzed. The following day the chitin was precipitated by the addition of approximately 140 ml. of 20 per cent potassium hydroxide. The precipitate was placed on a Büchner funnel and washed repeatedly with distilled water until the filtrate was neutral to litmus. Thirty-five grams of the chitin, as removed from the filter, were then added to agar which contained 0.1 per cent glucose, 0.1 per cent peptone, and 0.005 per cent dipotassium hydrogen phosphate. After autoclaving, the agar was thoroughly shaken to redistribute the chitin, and Petri plates were prepared. In this condition the medium was of a uniform milky-white opacity. When chitin-decomposing bacteria grew on the surface of this agar they dissolved the chitin, thereby producing a clear halo around each colony. By noting the length of time necessary for the appearance of halos the rates of bacterial activity could be estimated. Bacteria which decomposed chitin rapidly often produced a halo after two days' growth whereas others required eight to ten days for a positive test. Although bacteria which were

unable to decompose chitin often grew well on the medium, they did not alter it in appearance. Results obtained with this agar confirmed, without exception, the results obtained with other solid and liquid media.

In order to test a given sample of marine material for chitin-decomposing bacteria, several different media were inoculated. The inoculated media were incubated at room temperature and examined periodically for evidence of chitin breakdown. Growth was slow in the medium consisting of only chitin in sea water, whereas the same bacteria decomposed the chitin more rapidly when additional nutrients were present.

Visible dissolution of the chitin, either in liquid medium or on a solid agar surface, was indisputable evidence that the chitin was being attacked. To detect incipient decomposition a hand lens was helpful. The liberation of ammonia from media lacking peptone or nitrate, reducing substances from media lacking glucose, or a pronounced change in acidity, were further reliable evidence of chitin decomposition. The mere growth of bacteria in a medium consisting of only chitin in sea water is an unreliable criterion of decomposition, since bacteria may persist in this medium for a long period without altering the chitin.

In preliminary studies, cultures of bacteria showing no evidence of chitin-decomposition were kept one year before being discarded as negative. It became evident, however, that several weeks were adequate for detecting the chitin-decomposing capacity of most species. To obtain pure cultures of bacteria a second tube of enriched medium was inoculated with a loopful of material from an original positive culture. Growth from this tube was then streaked on nutrient agar plates and the different types of colonies which developed were retested for their ability to attack chitin. In later experiments pure cultures were obtained more quickly by streaking growth from enriched cultures on agar medium containing precipitated chitin. This medium was highly satisfactory for separating the chitin-decomposing bacteria from others which had persisted but which were not attacking the chitin.

Occurrence of Chitin-Decomposing Bacteria in the Sea

The number of chitin-decomposing bacteria in sand, mud, and water was determined by dilution culture methods. To increase the probability of detecting chitin-decomposing bacteria, several kinds of culture media were inoculated with each dilution of the sample. In making quantitative determinations only visible breakdown of the chitin was considered a positive test.

An 11 cm. core of sand, taken from a littoral zone in the vicinity of Woods Hole harbor on July 31, 1939, was found to be a relatively rich source of chitin-decomposing bacteria. At the top of the core there were at least 60,000 of these bacteria per gram of sand, this number decreasing to 6,000 per gram at a depth of 5 cm., and to 600 per gram at 11 cm.

A sample of mud was collected on July 7, 1939, at latitude 40° 38′ N. × 71° 39′ W. and at a depth of 878 meters. Chitin-decomposing bacteria were relatively abundant in the upper layer of the core (125 cells per gram), but dropped to less than 5 cells per gram at depths of 5 and 11 cm. Both in sand and in mud the number of chitin-decomposing bacteria decreased sharply with core depth.

On August 2, 1939, a sample of water was collected at 5 fathoms NE. of Lambert's Cove, Martha's Vineyard, one mile offshore, in a locality where the water is naturally well stirred. Dilution culture methods indicated the presence of 150 bacteria per ml. of water.

Chitin-decomposing bacteria were isolated from other samples of sand, mud and water, in addition to those described above. There was no correlation noted between abundance of the bacteria in the sediments and depth of the overlying water. In addition to bacteria isolated from media collected in the vicinity of Woods Hole a few cultures of chitin-decomposing bacteria were isolated from samples of water brought from tropical regions by the research ship "Atlantis."

Dead and decomposing horseshoe crabs offered an excellent source of chitin-decomposing bacteria. A single attempt to isolate these bacteria from a jar of dead copepods was unsuccessful.

That animals may possess intestinal fauna and flora capable of breaking down certain foods ingested by the host is well established. It has been demonstrated, for example, that wood-boring termites harbor in their intestinal tract microörganisms which perform for their host the invaluable function of digesting cellulose (15). Since a similar condition in relation to chitin might exist in marine organisms, the intestinal contents of several common marine animals were examined for chitin-decomposing bacteria. Using methods already described for their detection, chitin-decomposing bacteria were found in the intestinal contents of *Venus mercenaria mercenaria*, *Ostrea virginica*, *Loligo pealeii pealeii*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus*, *Raia erinacea* and *Spheroides maculatus*. In this investigation, all animals whose intestinal contents were examined for chitin-decomposing bacteria were found to possess them. The presence of these bacteria in the intestines of marine animals has previously been demonstrated by Benton (1), who isolated them from the speckled

trout and by ZoBell and Rittenberg (17), who found from one hundred to more than a thousand chitin digesters in each milliliter of the stomach contents of the squid.

There are few marine animals which do not ingest chitin if they come in contact with it. According to available information (8, 9, 10), however, the oyster does not take in chitin at any time. Accordingly, it is interesting to note that chitin-decomposing bacteria were found in the alimentary canal of this animal, also.

Decomposition of Limulus Shell in Natural Media

Having demonstrated the presence of chitin-decomposing bacteria in marine habitats, it was decided to follow the breakdown of a naturally-occurring chitinous material in several marine media. Accordingly, *Limulus* shells were scrubbed free of flesh, cut into strips approximately 3 × 8 cm., weighed (about 3 grams after drying overnight at 100–105° C.) and placed in marine sand, mud and sea water. Sand from the littoral region was scooped up, to a depth of 15 cm., and thoroughly mixed. Weighed strips of crab shell were placed in glass jars and covered with 200 grams of the sand. Likewise, strips of *Limulus* shell were covered with bottom mud collected in the Woods Hole harbor. Sea water, collected a few feet from the shore, was placed in bottles in 200 ml. volumes. Weighed strips of crab shell extended about 1 cm. above the water level. All cultures were kept in a dark cabinet at room temperature. Occasionally distilled water was added to the sand and mud cultures to prevent them from becoming dry.

These experiments were continued for 57 weeks. At stated intervals one culture from each of the three series (sand, mud, water) was examined. The crab shell was removed, weighed and checked for visible evidence of decomposition. Any changes in color and texture of the natural media were noted. The sand and the mud were analyzed for total organic carbon and nitrogen. The carbon was determined by Tiurin's modification (13) of Schollenberger's method (11). The nitrogen determinations were made by the usual Kjeldahl procedure. The results of the analyses are based upon the total dry sand or mud (dried overnight at 100–105° C.) not freed from the sea salt.

In sand, decomposition of the *Limulus* shell began at once. At the end of one year, when the experiment was discontinued, the shell had lost about three-fourths of its original weight (Fig. 1) and had been reduced to a small fraction of its former size. At the beginning of this experiment the sand contained 0.8 per cent carbon, and 0.05 per cent nitrogen, indicating that its organic content was relatively low. Al-

though a large amount of organic material was decomposed during the course of a year, there was no measurable increase in carbon and nitrogen in the sand. Presumably the compounds liberated from the crab shell were used in the metabolism of the bacteria.

When *Limulus* shell was placed in bottom mud, decomposition of the shell, as measured by decrease in weight, was detectable only after a period of about six weeks. Thereafter the shell decomposed fairly rapidly until at the end of one year it had lost a third of its weight (Fig. 1). The original mud was high in organic content. When brought into the laboratory it contained 2.4 per cent carbon and 0.17 per cent nitrogen. During the course of decomposition of the crab

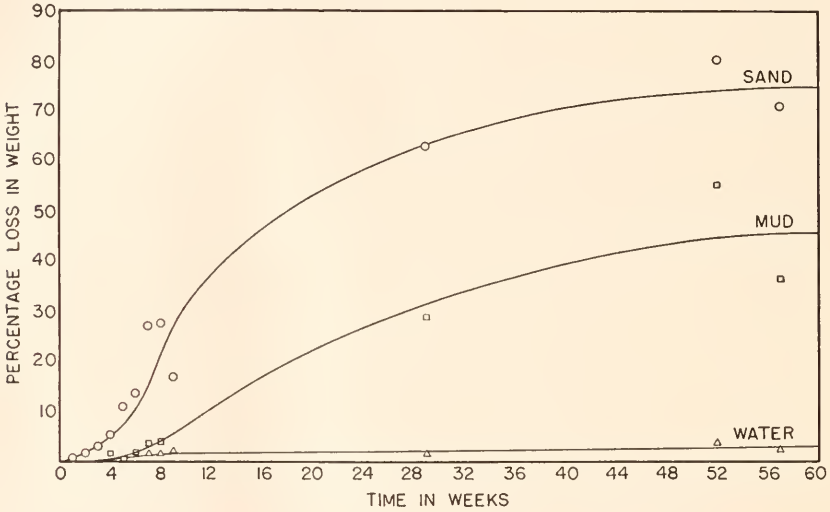

FIG. 1. Decomposition of horseshoe crab shell in natural marine media.

shell over a period of one year, the amount of carbon in the mud remained unchanged, while the nitrogen increased from 0.17 to 0.22 per cent.

In sea water there was only a very slight decrease in weight of the crab shell, even after a period of more than a year (Fig. 1). At the end of this time the shell was practically unchanged in appearance. This need not mean that chitin-decomposing bacteria were absent from the sea water, but only that under the experimental conditions there was no marked breakdown of the chitin in this medium, whereas in sand and in mud, under comparable conditions, the decomposition was pronounced. As far as laboratory experiments can show, chitin appears to be decomposed more slowly by marine bacteria than are most other structural organic materials.

During decomposition the shell underwent striking physical changes. Originally tough and leathery, it became soft and fragile as large areas were removed. Incipient decomposition was detected by using a binocular microscope, when the shell was observed to be "eaten" away in spots. In advanced stages of decomposition the shell was greatly reduced in size as well as in weight. In preparing purified chitin from *Limulus*, it was noted that continuous sheets of the purified material were obtained from all parts of the shell. It follows, therefore, that as the latter decomposed in these natural marine media, the chitin was digested along with the other compounds which make up the horseshoe crab shell.

The detailed characteristics of these bacteria are now being investigated. Preliminary tests indicate that approximately fifteen types of bacteria have been isolated. They are Gram negative, asporogenous, motile rods. Among the readily demonstrable products resulting from the decomposition of purified chitin by pure cultures of the bacteria are ammonia, reducing substances, and organic acids.

Summary

Chitin-decomposing bacteria were isolated from the following sources: marine sand, mud, water, decomposing horseshoe crabs, and the intestinal contents of *Venus mercenaria mercenaria*, *Ostrea virginica*, *Loligo pealeii pealeii*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus*, *Raia erinacea* and *Spheroides maculatus*.

Chitin-decomposing bacteria were found to be most abundant in marine sand, less abundant in mud, and present in small numbers in sea water.

Under experimental conditions, the decomposition of *Limulus* shell was relatively rapid in littoral sand, moderate in bottom mud, and negligible in sea water.

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